ORIGINAL ARTICLE

COMPARATIVE EVALUATION OF REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION AND IMMUNOHISTOCHEMISTRY FOR THE DIAGNOSIS OF PORCINE EPIDEMIC DIARRHEA IN LUZON, PHILIPPINES

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ABSTRACT

Reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) were used to demonstrate the presence of porcine epidemic diarrhea virus (PEDV) antigen in the intestinal cells of suckling pigs in Luzon. The PEDV antigen was detected in 11 (13.75%) and 32 (40%) out of 80 intestinal samples using RT-PCR and IHC, respectively. RT-PCR generated a 412-bp cDNA probe which amplified the viral RNA encoding the membrane protein of PEDV from the intestinal segments of the jejunum. Immunohistochemistry revealed positive cells in the jejunum as indicated by the brown staining in the cytoplasm of infected cells. Comparative evaluation of the two tests revealed a fair agreement. Histopathological changes observed include vacuolation of enterocytes, villous atrophy as exemplified by 2:1 villous:crypt height ratio and exfoliation of enterocytes which are associated with the clinical signs of PED such as watery diarrhea, dehydration and acidosis. RT-PCR may be used as a screening test for PEDV antigen detection using jejunal tissue with feces because of the shorter duration of processing and testing. IHC, on the other hand, can be performed as confirmatory test using formalin-fixed jejunal samples.

Keywords: histopathology, immunohistochemistry, porcine epidemic diarrhea, RT-PCR, swine

INTRODUCTION

Porcine epidemic diarrhea (PED) is a viral enteric disease of swine caused by a single stranded, positive sense, enveloped RNA virus with approximately 28 kb viral genome called Porcine Epidemic Diarrhea Virus (PEDV). PEDV is categorized under the genus Alpha coronavirus, family Coronaviridae, order Nidovirales (Pan *et al.,* 2012). Transmission occurs via feco-oral route after the introduction of infected animals or contaminated materials (Carvajal *et al.,* 1995). The disease is characterized by vomiting, watery diarrhea and dehydration in all ages of swine and manifests as sporadic outbreaks (Song *et al.,* 2012) causing significant economic losses in the swine industry. PED has been reported to cause a 100% morbidity and mortality rate in less than 5 days old neonatal piglets and 10% mortality in infected piglets older than 10 days old (Kim and Chae, 2002).

Methods for detection of PED infection are necessary prerequisite for assessing the current epidemic situation in herds and for subsequent immunoprophylactic measures (Roda'k *et al.,* 2005). Many diagnostic methods have been used for the detection of

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PEDV such as immunofluorescence tests, direct electron microscopy, enzyme linked immunosorbent assays (ELISA), *in situ* hybridization, immunochromatographic assay kits, reverse trancriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC).

In the Philippines, the immunochromatographic assay kit, immunofluorescence antibody test and RT-PCR using different primers are used to detect PED. However, immunochromatographic assay kits have been reported to be less accurate in detecting the disease with only 92% sensitivity (Song *et al.,* 2012). Employing highly sensitive, specific, more reliable and rapid methods of detection of PEDV-specific nucleic acid in fecal and intestinal samples such as RT-PCR and in formalin-fixed tissues for IHC, would be a useful and accurate method for the detection of PEDV antigen. This will also strengthen the disease reporting in the country which is important for disease control.

The study aimed to demonstrate the presence of PEDV antigen in naturally infected pigs using RT-PCR of intestinal samples and immunohistochemistry (IHC) in formalin-fixed paraffin-embedded intestinal tissues, determine the level of agreement of RT-PCR and IHC in the diagnosis of PED and to describe the histopathological lesions in the jejunum of PED infected piglets.

MATERIALS AND METHODS

Sample collection

Intestinal samples were collected from 80 diarrheic piglets, 4-6 days old, from a cooperating commercial farm in Southern Luzon. A one inch long section of the jejunum was collected from each piglet and placed in 10% neutral buffered formalin for immunohistochemistry and histopathological evaluation while the remaining jejunal and ileal segments were saved in plastic bags and kept in ice box during transport for the RT-PCR. Samples were then frozen at -20°C for long term storage.

Reverse transcriptase–polymerase chain reaction

One gram of minced small intestinal sample was mixed with 1 ml of phosphate-buffered saline (0.1 M, pH 7.2). The suspension was clarified at 9000 × g for 10 min at 4°C. Five hundred microliters of supernatants were added with 500 µl of Trizol LS Reagent. The samples were homogenized by vortexing and incubated for 5 min to permit complete dissociation of the nucleoprotein complex. A 300 µl of chloroform was added and the sample was vortexed for 2 min followed by incubation for 10 min at 4°C. The samples were then centrifuged at 4000 × g for 15 min at 4°C. The mixture separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA remains at the aqueous phase exclusively. The aqueous phase was removed by angling the tube at 45° and pipetting the solution out. The solution was placed in a new tube wherein 100% isopropanol was added for precipitation which was carried out overnight at 4°C. The precipitated RNA was pelleted by centrifugation for 10 min at 14000 × g and the supernatant was discarded. The pellet was washed with 75% ethanol and air-dried. The RNA pellet was resuspended in 30 µl of RNAse-free water by passing the solution up and down several times through a pipette tip. The RNA suspension was incubated in a water bath set at 55°C for 10 min.

The set of primers described by Kim *et al.* (2000) was used in this study. It amplifies the 412 bp region from the membrane protein gene of PEDV. The forward and reverse primers were 5'-GGGCGCCTGTATAGAGTTTA-3' (nucleotides 927–946) and 5'-AGACCACCAAGAATGTGTCC-3' (nucleotides 1319–1338). The commercial one-step RT-PCR system kit (Promega AccessQuick™, Promega Biotech, Madison, Wisconsin, USA) was used in this study. Twenty micromoles of the master mix, 2.5 µl each of the forward and reverse primers, and 5 µl of the RNA template were mixed and brought to a final volume of 50 µl with nuclease-free water. A 1 µl of the AMV Reverse Transcriptase was added to the mixture as the final component by vortexing. The reaction tubes were incubated at 45°C for 45 min in a water bath. PCR amplification was initiated at 94°C for 2 min and 40 cycles of denaturation at 94°C for 30 sec, primer annealing at 48°C for 30 sec and extension at 72°C for 30 sec. The final extension step was done at 72°C for 10 min. The amplified RT-PCR products were visualized by standard gel electrophoresis of 15 µl of the final reaction mixture on a 2% agarose gel. Amplified cDNA fragments were located by ultraviolet fluorescence after staining with ethidium bromide.

Immunohistochemistry

Tissue sections from the jejunum of each piglet were collected, fixed in 10% neutral buffered formalin and embedded in paraffin. The specimens were sectioned at 4 µm thickness. Samples were processed for the routine Hematoxylin-Eosin technique for the histopathological description. The specimens for immunohistochemistry were placed on poly-L-lysine coated glass microscope slides. Tissues were dehydrated in an oven at 60°C for an hour and deparaffinized with xylene, rehydrated with graded alcohols and washed with distilled water.

Enzymatic antigen retrieval method was carried out for 10 min at 30°C. Succeeding immunohistochemistry procedures followed the manual staining protocol of the Dako Envision® + Dual Link System- HRP DAB+ (Dako Denmark A/S). Tissue samples were incubated with the monoclonal antibody in antibody diluent at 1:100 dilution for 1 hour at 4°C. Tissue samples were counterstained with hematoxylin for 5 min followed by the rehydration steps and mounting. All incubations were done on slides placed horizontally on a thermal plate at 4°C. After each incubation, the slides were dipped in Tris-buffered saline with Tween-20 (TBST) for washing.

Routine histopathological examination

Jejunal samples were processed using the routine paraffin technique and stained with Hematoxylin-Eosin stain. The lesions were described in terms of presence or absence of vacuolation of enterocytes, villous atrophy and exfoliation of enterocytes: sloughing off of the epithelial cells covering the villi. Average villous lengths and crypt depths were determined in selected areas in which the villi and crypts are continuous and sectioned through their entire length. At that point, the mean length of the three longest villi and mean depths of three adjacent crypts were determined using an ocular micrometer.

Diagnostic comparison

The diagnostic performance of the two tests used for the detection of PEDV antigen field samples were compared and the level of agreement between RT-PCR and IHC in detecting PED infected animals was computed using the kappa-test to determine the % agreement. Presently, there is no "gold standard" diagnostic method for detecting PED-infected animals. Gold standards are established by the OIE. As a result, pigs were considered infected if they tested positive by RT-PCR or by IHC in the intestinal sections. Data were encoded in Microsoft Excel 2010 and analyzed using the Win Episcope 2.0.

RESULTS AND DISCUSSION

Eighty 4-6 day old diarrheic piglets were necropsied and examined in parallel to demonstrate the presence of PEDV antigen in the fresh and formalin-fixed jejunal segments of each piglet for RT-PCR and IHC respectively. Out of the 80 samples examined, 11 samples (13.75%) were positive for the PEDV antigen using RT-PCR and 32 samples (40%) using IHC. Physical examination showed piglets with emaciation, dehydration, anemia evidenced by pale mucous membrane, rough hair coat and soiling of perianal area with yellowish fecal material. Some of the piglets for necropsy were also observed vomiting. Examination of the gut revealed thin-walled, fluid and gas-filled yellowish to greenish small intestines with multifocal congestions. These findings coincide with the gross morphologic descriptions made by Sueyoshi *et al. (*1995); Lee *et al. (*1999) and Pensaert and Yeo (2006).

Figure 1 shows the 412-bp cDNA probe (Lanes 3, 7 and 10) generated by RT-PCR for the viral RNA encoding the membrane protein of PEDV from the intestinal segments of the jejunum. The presence of the band (amplicon) in the 400bp row indicates the presence of PEDV antigens in the intestinal sample. The relatively small number of positive results generated by the RT-PCR technique (11/80) may be attributed to a possible low number of virus present in the samples. Ishikawa *et al.,* (1997) reported that 100 $TCID_{50}$/sample viral load can be detected within 8-hr post-infection by RT-PCR. The report made by Pensaert and Yeo (2006) wherein viral shedding of PEDV infection occurs within 4-5 days post-infection can also explain the low number of positive results in this study since the piglets are 4-6 day old and it can be assumed to be in the stage of shedding the virus when the intestinal collection was done. Sozzi *et al.,* (2010) reported that RT-PCR detection using intestinal samples yielded a lower number of positive detections as compared when fecal samples alone were used. The assay could also have been affected by the presence of inhibitors in the intestinal and fecal samples although the mechanism of the inhibition is unknown but may be related with the variable amounts of bilirubin and bile salts which inhibit the thermostable activity of the DNA polymerase (Kim and Chae 2002).

Examination of the jejunal tissue samples placed on poly-L-lysine coated glass microscope slides for immunohistochemistry revealed the presence of brown staining in the cytoplasm of the cells in the sloughed-off mucosa (Figure 2), lamina propria and macrophages in the villi of the jejunum. The presence of viral antigens as indicated by the brown-staining in the cytoplasm of positive villous epithelial cells can be explained by the pathogenesis of PED wherein PEDV enters the cell via binding to the aminopeptidase N receptor and after which viral replication occurs through intracytoplasmic budding in the cytoplasm of infected cells (Pensaert and Yeo, 2006). The result of the IHC technique of this study agrees with the findings of the study done by Sueyoshi *et al.* (1995); Guscetti *et al.* (1998); Kim *et al.* (1999); Lee *et al.* (2000) and Kim and Chae (2002) wherein positive brown staining areas in the cytoplasm of the lining epithelial cells, lamina propria and macrophages in the jejunal villi were observed. Also, no positive-staining cells were detected in the crypts of Lieberkühn of the samples in the present study. This observation is similar with other studies (Kim *et al.,* 1999).

The light brown staining in the cytoplasm of positive cells as compared to the other immunohistochemical studies can be explained by the use in the present study of a monoclonal antibody which is highly specific for a single sequence or “epitope” of the antigen molecule (Ramos-Vara *et al.,* 1999). These findings may also indicate a low membrane protein load in these parts of the enterocytes (Kim *et al.,* 1999). The results of

this study agree with the results of Kim and Chae (2002) who found positive enterocytes distributed in the jejunal samples. In this study, positive staining cells were mostly found in the sloughed-off cells of the lining epithelium, lamina propria and in some macrophages of the villi of the jejunum. These results agree with that of Lee *et al.,* (2000) who reported that most of the infected cells were not continuously arranged in the epithelial layer and the borders between infected cells were distinct. The number of viral antigens could also vary depending on the stage of the disease process because infected enterocytes tends to decrease with increasing severity of villous atrophy which may be either caused by PEDV leaving the enterocyte or caused by erosion of the infected cells (Sueyoshi *et al.,* 1995).

Test results are summarized and plotted in a 2 x 2 table as shown in the Table. It

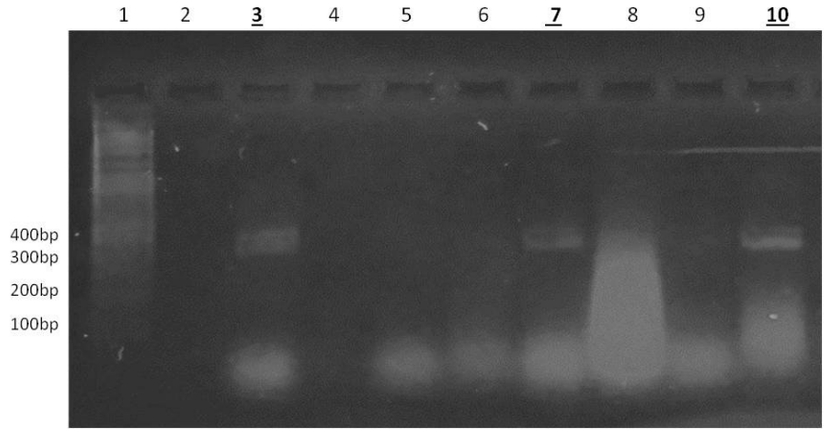

Figure 1. Ethidium bromide stained RT-PCR products after electrophoresis. Lanes 3, 7 and 10 shows the positive band amplifying the 412-bp M protein of PEDV with Lane 1 as the reference marker.

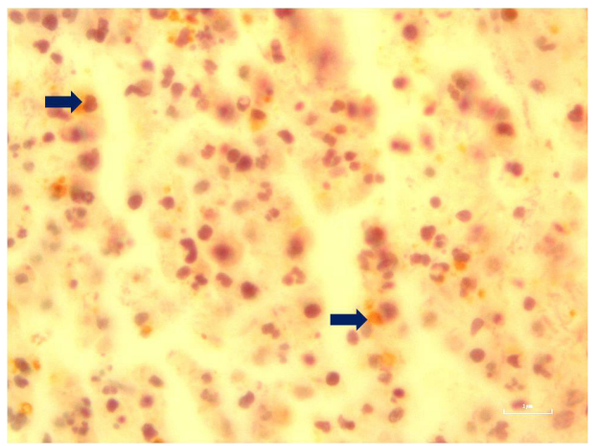

Figure 2. Sloughed-off villous enterocytes in the jejunal lumen showing brown-staining cytoplasm of cells positive for porcine epidemic diarrhea virus antigen (blue arrows).

can be noted that out of the 80 animals examined, 10 were positive in both RT-PCR and IHC for the PEDV antigen and it can be assumed that a higher probability of being truly positive with the PEDV antigen is possible in these animals. On the other hand, 47 animals turned negative in both RT-PCR and IHC and can be assumed that these animals have higher chances of being truly free of the PEDV antigen as detected by both tests. Also, out of the 48 piglets negative for IHC, one piglet was positive with the PEDV antigen using RT-PCR. This result can be attributed to the absence of PEDV antigen in that specific section of the jejunum.

Kappa test of the comparison of diagnostic performance of RT-PCR and IHC yielded a kappa value of 0.327 at 95% confidence (Win Episcope 2.0 Test Agreement Module). This numerical value of kappa is interpreted as fair agreement according to Everitt as cited by Trushfield (2005). However, this does not coincide with the results of Kim *et al.,* (2002) who reported a 0.83 agreement, interpreted as almost perfect agreement among RT-PCR, IHC and *in situ* hybridization. Sozzi *et al.* (2010) in their study regarding the comparative evaluation of RT-PCR and ELISA in the detection of PEDV, reported a substantial agreement (0.62) between the two tests.

The low level of agreement between RT-PCR and IHC may be attributed to the failure of RT-PCR to detect very low viral load (Sozzi *et al.,* 2010) or the very specific binding of the monoclonal antibody used to the PEDV antigen (Ramos-Vara *et al.,* 1999). The time of collection may also be considered wherein the sample animals are in the recovery phase of the disease or shedding the virus when clinical samples were collected (Sozzi *et al.,* 2010). This will lead to fewer villous intestinal cells available for the attachment of the virus.

The fair agreement between RT-PCR and IHC could indicate that only one test is good, or that both tests are bad, or that both tests are good but negatively correlated which can occur in some antibody and antigen tests. The kappa value depends on the prevalence of the disease (Trushfield, 2005). Majority of recent studies in PEDV infection makes use of the RT-PCR as the diagnostic tool wherein fecal samples are commonly used rather than intestinal samples because of the ease of collection without sacrificing the animal. Also, most of the studies done with regards to PED is geared towards identifying the phylogeny of the virus strain present in one country since this would be beneficial in the development of vaccines against this disease. In this study, IHC detected a greater number of animals positive for the PEDV antigen. IHC provides opportunity for diagnosis when only formalin-fixed samples are available.

Histopathological examination of the intestinal tissues of piglets positive in either RT-PCR or IHC technique revealed severe sloughing-off of the enterocytes in the lining

Table. Comparison between results obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) tests for diagnosis of porcine epidemic diarrhea virus.

		IHC		Total
		+	-	
RT-PCR	**+**	10	1	11
	-	22	47	69
	Total	32	48	80

epithelium of the jejunum and blunting of the villi (Figure 2). The intestinal lining epithelia normally lined by absorptive simple columnar cells become cuboidal or low columnar with indistinct brush border (Sueyoshi *et al.,* 1995). Villous intestinal cells present small cavities in the cytoplasm with pyknotic nuclei displaced at the periphery and some of which have lost their nuclei. Blunting of intestinal villi was so severe leading to sloughing-off of the intestinal epithelium and villous atrophy as determined by the villi crypt-ratio which was found to be 2:1 in this study. This ratio is far different from the normal villi: crypt ratio which is 7:1 (Pensaert and Yeo, 2006) and is lower than with the findings of Lee *et al.,* (2000) which is 3:1. A slight lymphocyte infiltration was also observed. These histopathological changes present in this study coincide with the descriptions done by Lee *et al.,* (2000) which includes vacuolation of enterocytes, villous atrophy and exfoliation of enterocytes.

The watery feces observed in PEDV infection in the 4-6 day-old age group in this study can be explained by the increased susceptibility of the sucklings to PEDV infection due to their less acidic gastric secretions and their milk diet acts as buffer for the gastric acid which protects the virus during its passage to the small intestines (Schwartz and Main, 2013). The PEDV replication and assembly in the cytoplasm of the villous cells lead to the degeneration of the cells in the lining epithelium of the small intestines (Pensaert and Yeo, 2006). The loss of the absorptive epithelium and the regeneration of these cells by the secretory cells in the crypts results in the net secretion of water and electrolytes. The presence of incompletely absorbed nutrients and undigested milk all contribute to the occurrence of watery diarrhea (Kumar *et al.,* 2005). Dehydration occurs as a consequence of excess fluid loss in the feces which results to hypovolemia leading to inadequate tissue perfusion because of hemoconcentration. In this case, energy is generated through anaerobic glycolysis causing hypoglycemia and later on to acidosis (Zachary and McGavin, 2012).

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